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Reduced expression of BMP-3 due to mechanical loading

A link between mechanical stimuli and tissue differentiation

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ABSTRACT – Mechanical signaling and BMP expression appear to be involved in controlling the differentiation of cartilage in fracture repair, but the connection between mechanics and BMP signaling is not known. In this study of rats, we used a bone chamber to see how BMP gene expression was changed by a mechanical loading regime that induces cartilage formation in this model. We compared the still undifferentiated tissue in loaded and unloaded chambers in the same rat regarding the expression of TGFß-1, BMP-2, 3, 4, 5, 6, 7, CDMP-1, 2 and ALK-2 and 3 by using RT-PCR normalized against GAPDH. We found expression of TGFß-1, BMP-2 and 4 in all specimens, and BMP 5-7 and CDMPs in none. 1 week after loading started, BMP-3 was strongly expressed in the unloaded control specimens in 7 of 8 animals, but detectable in only 1 of the contralateral loaded ones. After 2 weeks of loading, the BMP-3 expression pattern was less clear, but with both time groups taken together, there was still less BMP-3 expression on the loaded side in 9 rats, more in 1 and no difference in 5 (p = 0.01). ALK-2 at 1 week was expressed in all specimens expressing BMP-3 and in none of the others. At 2 weeks, ALK-2 was expressed in all specimens. Thus, a loading regime, known to induce cartilage in this model, caused down-regulation of BMP-3 and ALK-2. The results are consistent with the view that BMP-3 inhibits differentiation, as recently described. This role appears to be linked to the ALK-2 receptor. Most importantly, the results indicate a link between mechanical signaling and BMP expression such that mechanically-induced down-regulation of the inhibiting BMP-3 enabled the induction of cartilage.

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Cartilage formation during, e.g., fracture repair, starts in a mass of undifferentiated mesenchymal cells, a blastema. Cartilage differentiation is controlled by several members of the TGF-ß superfamily, notably the BMPs (Bostrom et al. 1995, Kingsley 1998, Merino et al. 1998). The process is also influenced by mechanical stimuli. The theory of Pauwels (1960), later refined by others (Carter et al. 1998), predicts that local increases in hydrostatic pressure cause cartilage differentiation within a blastema. Most studies of this theory have been performed by computer simulation, using finite element models. Recent comparisons between animal models and computer simulations reveal that cartilage induction occurred in regions with a hydrostatic pressure exceeding 0.15 MPa and with less than 15% strain (Claes et al. 1998). Cartilage induction by applying hydrostatic pressure has been shown in a model in which only membraneous ossification otherwise would occur (Tägil and Aspenberg 1999). The relation between mechanical signaling and morphogen expression is unknown. BMPs may have different effects in mechanically different environments. For exam-

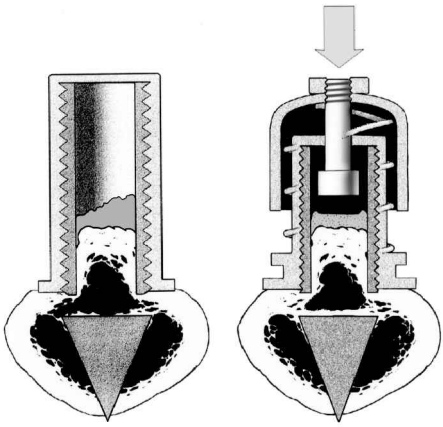

Figure 1. Transverse sections of the tibia with the bone conduction chamber (BCC) to the left and the load chamber (LC) to the right. In the BCC, the ingrowing tissue is not mechanically stimulated due to the stiff titanium walls. The loading force (arrow) is applied to the LC by a dynamometer from outside the skin and transferred to the piston and the ingrowing tissue. Bone is shown in yellow and soft tissue in red. A spring keeps the piston in its upper position when not loaded (Partly reproduced with permission from J Orthop Res 1998; 17 (2): 201).

ple, the potent bone-inducer BMP-2 inhibited or totally abolished bone repair in a bone chamber model in rabbits (Jeppsson et al. 1999), but when a small mechanical stimulus was added, the effect switched from inhibition to stimulation of bone formation (Bostrom et al. 1998). Thus, mechanical stimuli can direct a developmental process initiated by a BMP. Conversely, and perhaps more often, mechanical stimuli can influence the expression of various BMPs and their inhibitors. To date, however, no mechanical effect on BMP expression in vivo has been shown, except perhaps during distraction osteogenesis (Sato et al. 1999). To find a link between mechanical stimuli and BMP signaling, we measured the expression of several BMPs in a model for mechanical cartilage induction. The measurements were made in the blastema 1 or 2 weeks after loading started.

Material and methods

2 different chambers were used. The unloaded bone conduction chamber (BCC; Figure 1A) consists of a threaded titanium cylinder formed of 2 half cylinders held together by a closed screw cap. One end of the implant is screwed into the bone. The interior of the chamber has a diameter of 2 mm and is 7 mm long. 2 ingrowth openings at 1 end allow tissue to grow in from the subcortical cancellous bone. The load chamber (LC; Figure 1B) consists of the BCC equipped with a 1.8 mm diameter piston protruding into the chamber from its subcutaneous end towards the intraosseous end. The upper part of the piston, which is outside the chamber, is connected to a subcutaneous cap. The whole construct is easily palpable through the skin and by pressing through the skin against the cap, the piston can be moved downwards into the chamber with a known force. After pressurizing, the piston returns to its upper position by means of a spring.

After implantation, the BCC is normally invaded by undifferentiated mesenchymal cells and capillaries advancing from the ingrowth holes towards the subcutaneous end. At 3 weeks, this tissue has usually reached 1.5–2.0 mm into the chamber and at 6 weeks, 2.5–3.0 mm. Behind this advancing borderline of fibrous or undifferentiated mesenchymal tissue is a second one at which the tissue becomes ossified by membranous ossification. Behind the ossification borderline is a third one at which the bone is resorbed forming marrow cavity. Thus, from 3 weeks and onwards, an unloaded specimen contains from above to below: undifferentiated mesenchymal tissue, newly formed membranous bone and a marrow cavity surrounded by remodeled bone. In several hundreds of unloaded specimens in our laboratory, cartilage tissue has never been seen.

15 male Sprague Dawley rats (about 350 g) had a BCC implanted in one leg and a load chamber in the other. As in our previous study, the chambers were left unloaded during the first 3 weeks after implantation, to allow for tissue to grow in. Thereafter, loading was applied during 1 (n 8) or 2 (n 7) weeks, using the same protocol as previously. The load was applied by hand using a specially-designed dynamometer set at 8 N. This was held at the top of the load chamber outside the skin during 3 seconds, which was followed by an unloaded interval lasting another 3 seconds. This 6-second cycle was repeated 20 times twice a day. The stress exerted on the tissue beneath the piston was estimated at 2 MPa.

Table 1. Abbreviations

RT-PCR	Reverse transcriptase polymerase chain reaction
TGF-ß1	Transforming growth factor beta
BMP-2-6	Bone morphogenetic protein 2-6
BMP-7	Bone morphogenetic protein 7, also called Osteogenic protein 1 (OP-1)
CDMP-1, 2	Cartilage derived morphogenetic protein 1, 2; human correspondent to rat growth and differentiation factor 5,6 (GDF 5,6), also called BMP14,13.
ALK-2	Activin receptor-like kinase 2, also called Activin receptor I (ActRI)
ALK-3	Activin receptor-like kinase 3, also called BMP-receptor IA (BMPRIA)
GAPDH	Glyceraldehyde phosphate dehydrogenase, a "housekeeping" gene.

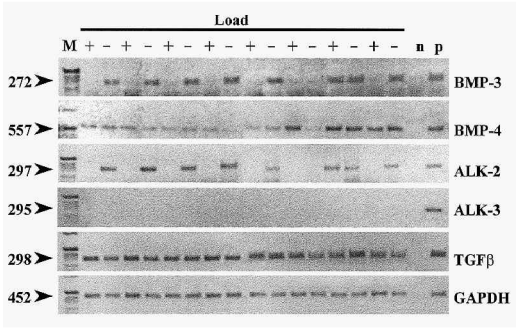

Figure 2. RT-PCR results showing the gene expression for each rat (plus and minus) after 1 week. The loaded side is shown by a plus sign and the unloaded by a minus sign. Staining for BMP-3 and ALK-2 seems to be less on the loaded side in 6 of 8 rats, indicating that these genes are down-regulated by load.

Evaluation

After 1 or 2 weeks of loading, the rats were killed with an overdose of pentobarbital. The specimens were harvested under sterile conditions and a 1 × 1 × 1 mm piece of the soft tissue adjacent to the piston was dissected and immediately frozen. The remainder of the soft tissue with the adjacent bone was then fixed in 4% formalin and prepared before routine histological examination with toluidine blue stain.

The frozen specimens were analyzed with RT-PCR for TGF-ß1, BMP-2, 3, 4, 5, 6, 7, CDMP-1, 2, ALK 2, 3 and normalized against GAPDH (Table 1).

RNA was isolated with TRIzol, as indicated by the manufacturer (GIBCO BRL, Gaithersburg, MD). Genomic DNA was removed with RNAse-free deoxyribonuclease (GIBCO BRL). Complementary DNA was synthesized with Superscript reverse transcriptase (GIBCO BRL). 4 µL of RNA were incubated with 2 µL oligoDT at 70 °C for 10 min. Each reaction contained 14 µL 5× buffer, 7 µL DTT (0.1 M), 3.5 µL of each nucleotide (10 mM), 1.5 µL RNase inhibitor (Boehringer Mannheim, Indianapolis, IN), and 2 µL Superscript reverse transciptase (GIBCO BRL). Samples were incubated for 10 min at room temperature, 50 min at 42 °C, 10 min at 50 °C, and 15 min at 75 °C. 1 µL RNase H (GIBCO BRL) was added to the reactions, and the samples were incubated for an additional 20 min at 37 °C. PCR was done, as indicated by the manufacturer (Perkin Elmer, Norwalk, CT) with the primers given in Table 1. Samples were incubated for 5 min at 95 °C, followed by 35 cycles for 45 s at 94 °C, 45 s at 58 °C and 1 min at 72 °C and a final extension at 72 °C for 10 min in a Perkin Elmer DNA Thermal Cycler. To compare the relative quantity of the RT-PCR reactions, the transciption level of GAPDH, a "housekeeping" gene was used as a control. PCR products were separated, using 1% agarose gels. Reactions without cDNA were used as a negative control, and rat embryonal cDNA as positive controls. All reactions were repeated at least twice.

Differences between the loaded and unloaded chambers were described as positive, negative or absent and assessed statistically with the sign test.

Results

All specimens showed expression of TGFß1 and BMP-2 and 4, whether loaded or not.

At 1 week, BMP-3 expression was present in 7/8 unloaded control specimens, but in only 1/8 contralateral loaded ones (Figure 2, Table 2.). There was a clear side difference in 6 rats and no difference in 2 ($p = 0.02$). At 2 weeks, BMP-3 expression was reduced by load in 3/7 rats and increased in one ($p = 0.01$ in both time groups analyzed together). ALK-2 at 1 week was seen in all specimens expressing BMP-3, but in none of the others. At 2 weeks, ALK-2 was expressed in all specimens (Table 2.).

Table 2

Rat	BMP-3		ALK-2		Load effect	
	L	U	L	U	BMP-3	ALK-2
1 week						
1	–	+	–	+	<	<
2	–	+	–	+	<	<
3	–	+	–	+	<	<
4	–	+	–	+	<	<
5	–	+	–	+	<	<
6	–	–	–	–	=	=
7	+	+	+	+	=	> [a]
8	–	+	–	+	<	<
2 weeks						
1	+	+	+	+	< [a]	=
2	–	–	+	+	=	=
3	+	–	+	+	>	=
4	–	–	+	+	=	=
5	–	+	+	+	<	=
6	–	+	+	+	<	=
7	–	–	+	+	=	=

L loaded, U unloaded.
The load effect column shows that for BMP-3, at both times, there is no difference in 5 cases, less on the loaded side in 9 cases and more on the loaded side in one case ($p = 0.01$). [a] indicates a definite difference, although there was expression in both samples.

BMP-5, 6 and 7, CDMP-1 and 2 and ALK-3 were not detected from any specimen, although these PCR products were obtained from the positive controls for all reactions.

On histological examinations, cartilage-like cells were seen in a few of the loaded specimens at both times, but all specimens mainly consisted of fibrous or undifferentiated tissue (Figure 3). Due to damage to the tissue when specimens for PCR were sampled, the histological sections were not sufficient for quantitative analysis.

Discussion

We found a change in the pattern of BMP expression by applying a load that is known to induce cartilage formation in this model (Tägil and Aspenberg 1999). BMP-3 and ALK-2 were the only genes that were not expressed in all specimens or none, and they were expressed in relation to loading. This makes a type one error due to mass-significance highly unlikely.

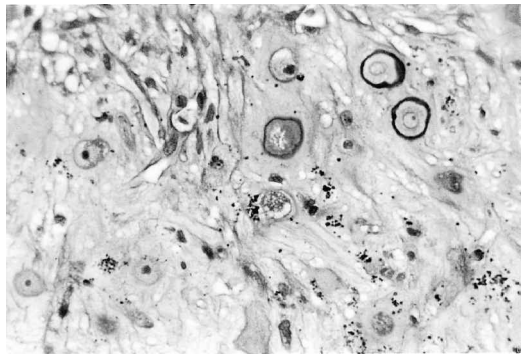

Figure 3. Soft tissue near the loading piston after 1 week of loading (Goldner). Some cartilaginous cells can be seen in the fibrous tissue, a phenomenon never found in unloaded chambers.

We believe that the changes in BMP-3 and ALK-2 expression occurred before cartilage was formed, and therefore be a cause of cartilage formation since, for reasons given below, the change in expression pattern must have occurred in the undifferentiated tissue. Most of the soft tissue on top of the bone in the chamber is fibrous or undifferentiated, and only a small portion is cartilage. The change in BMP expression must have take place in the fibrous or undifferentiated tissue, because a reduced BMP-3 expression occurring only in the cartilage would have been masked if there was continuing expression in the rest of the specimen. Further, the similarity in the expression of other BMPs, and the absence of CDMPs, known to be present in cartilage, indicate that the mRNA analyzed was derived from a non-cartilaginous tissue on both the loaded and the unloaded sides. This interpretation should be evaluated by using in situ hybridization.

The results relate to those of Daluiski et al. (1999), showing that BMP-3 is a negative regulator of osteogenesis in vitro and in vivo. They showed that BMP-3 inhibited alkaline phosphatase production induced by BMP-2 in vitro and that BMP-3 knock-out mice had about twice the trabecular bone volume as wild-type controls. Naturally-occurring BMP inhibitors, such as Noggin, act as soluble BMP receptors (Zimmerman et al. 1996). The inhibition by BMP-3 may result from the formation of inactive BMP-3 heterodimers with some other BMP, but also from a direct inhibitory effect of the BMP-3 homodimer.

ALK-2 and ALK-3 are type-I BMP receptors. The pathways upstream of BMP or BMP-receptor expression under these conditions are unknown. The effect of loading on ALK-2 expression disappeared during the second week. A a change in the mechanical environment (initiation of loading) may have affected ALK-2 expression, but once a response to this change had been made (triggering of differentiation), ALK-2 expression was normalized. Thus, the down-regulated ALK-2 expression appeared to reflect a need for a response rather than a loaded situation per se.

In conclusion, both the unloaded and loaded blastemas expressed TGFß1 and BMP-2 and -4. BMP-3 expression in the unloaded controls may have inhibited cartilage formation. Mechanical loading probably reduced BMP-3 expression, allowing BMP-2 and -4 to induce cartilage formation. This is the first description of a link between discrete mechanical stimuli and signaling proteins in the control of skeletal tissue differentiation.

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